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Polymerase

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<b>13. ABSTRACT (Maximum 200 Words)</b>  We have previously isolated an intact, stable, and fully functional multiprotein DNA replication complex (designated the DNA synthesome) from a variety of non-malignant and malignant tumor cells and tissues including breast cancer cells. We have also shown that poly(ADP-ribose) polymerase (PARP) is among the components of the DNA synthesome. The transformation of a non-malignant human cell to a malignant state is accompanied by a significant alteration in the mobility of specific components of the DNA synthesome, (such as PCNA) following 2D-PAGE analysis of the DNA synthesome, together with a 4-6-fold decrease in the replication fidelity of the replication complex. In order to establish whether the malignant transformation process is accompanied by an alteration in PARP, we purified PARP from malignant and non-malignant breast cells using phosphocellulose and hydroxylapatite chromatography. When analyzed by 2D SDS-PAGE, PARP isolated from the two cell lines showed a difference in the electrophoretic migration pattern. The enzyme present in non-malignant breast cells had a basic pI and was resolved as a single spot; however, malignant breast cell PARP appeared as a basic spot in addition to less abundant species having less basic pI values. The altered PARP isoforms were detected both in estrogen-dependent (MCF-7) and non-estrogen-dependent (MDA MB-468) cells. In addition, these isoforms were detected in ovarian cancer cell line PA-1. We have also found that PARP from malignant breast cells has different kinetic properties (Km and Vmax) than those of non-malignant PARP. Moreover, nuclear proteins in malignant and non-malignant breast cells are differentially poly(ADP-ribosyl)ated. The results presented in this report suggest that PARP might have a different role during malignancy.				
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## **INTRODUCTION:**

The genetic damage which accompanies the development and progression of breast cancer has been linked to defects in the DNA replication and repair processes in these cells. We have previously isolated an intact, stable and fully functional multiprotein DNA replication complex (designated the DNA synthesome) from a variety of non-malignant as well as malignant tumor cells and tissues including breast cancer cells. All of the components necessary for DNA replication, including poly(ADP-ribose) polymerase (PARP) have been detected in the DNA synthesome. We have shown that the malignant breast cell DNA synthesome exhibits a 4-6-fold decrease in replication fidelity relative to the non-malignant breast cell DNA synthesome (Sekowski et al., 1998). In addition, the transformation of a non-malignant human breast epithelial cells to a malignant state is accompanied by a significant alteration in the mobility of specific protein components of the DNA synthesome (such as proliferating cell nuclear antigen; PCNA) following two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of the replication complex (Bechtel et al., 1998). PARP is a nuclear enzyme of a molecular weight of 116,000. It plays an important role in DNA replication, repair, and recombination. It absolutely requires DNA single-strand or double-strand breaks for its enzymatic activity. This enzyme, once activated, catalyzes the transfer of ADP-ribose unit from nicotinamide adenine dinucleotide (NAD) to nuclear proteins such as histone and PARP itself. It has been shown that at least fifteen components of the DNA synthesome are poly(ADP-ribosylated), suggesting that PARP may play a regulatory role in controlling the activity and the fidelity of the DNA synthesome (Simbulan-Rosenthal et al., 1996 and 1998). The unique form of PCNA found exclusively in malignant breast cells lacks the poly(ADP-ribose) modification which is found in the non-malignant form of the protein. The goal of this project is to establish a link between the differences in PARP activity and the alteration(s) in structure exhibited by this protein in both malignant and non-malignant breast cells.

## **BODY:**

### **I. PARP was confirmed to be one of the Components of the DNA Synthesome:**

The DNA synthesome has been isolated and purified from breast cancer MDA MB-468 cells using a series of steps, which include centrifugation, polyethylene glycol precipitation, ion exchange chromatography, and density gradient sedimentation (Malkas et al., 1990; Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997) (Figure 1). P4 fraction (5 ml) containing approximately 30 mg of protein was loaded onto a Bio-Rad Q5 column pre-equilibrated with a buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 0.1% glycerol, and 1 mM EDTA. The column was then washed with the same buffer and eluted using a gradient of 50-500 mM KCl. One-milliliter fractions were collected. The eluted fractions were then dialyzed against a buffer containing 20 mM HEPES, pH 7.5, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mM DTT and aliquots of each fraction were stored at -80°C.

The P4 and Q-sepharose peaks were resolved through 10% denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis was performed using anti-human PARP antibody (BD Pharmingen). PARP was found to exclusively co-purify with the DNA synthesome (Figure 2).

Mass spectrometric analysis of the DNA synthesome enriched protein fractions also indicated that PARP is a part of the replication complex core. Each of the protein bands from SDS-PAGE was cut and analyzed by MALDI and Q-TOF mass spectrometry. It has been shown that PARP, PCNA, replication protein A (RPA), replication protein C (RFC), DNA polymerase  $\alpha$ , DNA methyltransferase, and FEN1 are components of the replication complex core (Dr. Suhua Han's 2001 Annual Report).

### **II. PARP Purification using Phosphocellulose and Hydroxylapatite Chromatography:**

In order to define the kinetic and physical characteristics of PARP, we conducted chromatographic procedures to purify that protein from malignant and non-malignant breast cells. We initiated experiments to purify PARP from the malignant MDA MB-468 and non-malignant MCF-10A cells according to the methods of Ushiro et al. (1987), Jump and Smulson (1980), and D'Amours et al. (1997). The purification procedures involved phosphocellulose followed by hydroxylapatite chromatography.

#### **1- Phosphocellulose Purification of PARP:**

The first step of PARP purification involved the use of a phosphocellulose column that dissociated the synthesome and resolved PARP from other proteins.

Cellulose phosphate (Sigma) was prepared according to the following protocol:

- 1- The resin was suspended in 5 volumes of distilled water overnight. It was stirred and allowed to settle for 45 minutes. The settled volume was measured and this

was considered the column volume (CV) required to measure the volumes of the washing solution.

- 2- The resin was suspended in 5 CV of 0.05 M NaOH containing 0.5 M NaCl for 10 minutes and the slurry was poured into a sintered glass funnel while applying gentle suction.
- 3- The slurry was washed with 5 CV of distilled deionized water.
- 4- The resin was suspended with 5 CV of 0.75 N HCl for 10 minutes and was poured back into the funnel. This was followed by passing fresh 0.75 N HCl through the bed.
- 5- The resin was washed with distilled deionized water using at least 9 CV until the effluent shows a pH=5.
- 6- The resin was packed into a column and equilibrated with **buffer A** containing 20 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl<sub>2</sub>, and 1 mM DTT.

10 grams of MDA MB-468 and MCF-10A cells were fractionated to the clarified nuclear extract (NE) according to our published protocol (Coll et al., 1996) (Figure 1A). The presence of PARP in the nuclear fraction (NE) was detected by SDS-PAGE (10%) and immunoblot analyses of 50 µg proteins of the homogenate (H), cytosolic (S1), and nuclear (NE) fractions. Most of PARP protein was detected in the NE fraction (Figure 3).

The NE fraction was dialyzed against **buffer A** and applied onto the phosphocellulose column equilibrated with the same buffer. PARP was eluted by a stepwise increase in NaCl concentration from 0.3 M to 0.7 M. 50 µg of NE protein fraction and 1 µg of the 0.3-0.7 M NaCl eluted fractions were resolved by 10% SDS-PAGE and PARP was detected by Western blot analysis using anti-human PARP monoclonal antibody. It has been found that most of the enzyme was eluted with 0.5 M NaCl (Figure 4). PARP has been greatly enriched during this step of purification.

### **2- Concentration of PARP Protein Present in the Phosphocellulose Peak:**

The phosphocellulose peak fractions enriched with PARP activity were concentrated using Centrifugal Filter Devices (Millipore) with a molecular weight cut-off of 50,000. The phosphocellulose peak was applied into the sample reservoir and concentrated according the instruction manual. 5 µl of concentrated fraction was then analyzed by 10% SDS-PAGE and PARP was detected by Western blot analysis using anti-human PARP antibody (Figure 5). PARP was further enriched after this step of purification.

### **3- Hydroxylapatite Purification of PARP:**

Purification of PARP using hydroxylapatite chromatography was carried out according the method of Jump and Smulson (1980).

Hydroxylapatite matrix (Fluka BioChemika) was prepared according to the following procedures:

- 1- Approximately one part hydroxylapatite matrix was added to six parts loading buffer (10 mM potassium phosphate buffer, pH 6.8) and resuspend by gentle swirling.
- 2- The slurry was allowed to settle for a minimum of 20 minutes and the fines were decanted.
- 3- The loading buffer was added to the settled bed and mixed gently by swirling. The matrix was allowed to settle.
- 4- After the matrix settled to give a sharp dividing line between the bed and the clear buffer above, it was resuspended by swirling and poured into the column.

NE fraction (MDA MB-468) was dialyzed against **buffer B**, containing 20 mM Tris-HCl pH 7.5, 10 mM potassium phosphate buffer, 1.5 mM  $MgCl_2$ , and 1 mM DTT, and applied onto the hydroxylapatite column equilibrated with the same buffer. PARP was eluted with a stepwise increase in potassium phosphate concentration (10-100 mM, pH 6.8). NE, flow throw (FT), and the potassium phosphate eluted fractions were resolved by 10% SDS-PAGE and PARP was detected by Western blot analysis. Most PARP protein was detected in the 500 mM potassium phosphate eluted fraction (Figure 6).

#### **4- Purification of PARP using a Combination of Phosphocellulose and Hydroxylapatite Chromatography:**

After establishing the conditions for hydroxylapatite purification of PARP, we combined both the phosphocellulose and hydroxylapatite chromatography techniques and PARP has been purified according the following procedures:

- 1- NE fractions from either MDA MB-468 or MCF-10A cells were prepared according to Coll et al. (1996). They were dialyzed against **buffer A** and applied onto phosphocellulose column equilibrated with the same buffer. PARP was eluted with increasing concentration of NaCl (0.3-0.7 M).
- 2- The phosphocellulose peaks (fractions eluted with 0.5 M NaCl, Figure 4) were dialyzed against **buffer B** and applied onto hydroxylapatite column equilibrated with the same buffer.
- 3- PARP was eluted with increasing concentrations of potassium phosphate buffer (25-1000 mM, pH 6.8).
- 4- NE, phosphocellulose peak, FT, and the potassium phosphate eluted fractions were resolved through 10% SDS-PAGE and PARP was detected by Western blot analysis.

Figure 7 shows that bands corresponding to PARP have been detected in the NE fraction and the phosphocellulose peak (0.5 M NaCl eluted fraction). A very faint PARP band was also detected in the hydroxylapatite fraction eluted with 500 mM potassium phosphate buffer.

In order to clearly detect PARP in the hydroxylapatite-eluted fractions, we concentrated these fractions using a speed-vac. The concentrated fractions together with the NE and the phosphocellulose peak were resolved by 10% SDS-PAGE and PARP was detected by Western blot analysis (Figure 8). In addition to detection of PARP bands in the NE and phosphocellulose peak, a clear band appeared in the 500 mM potassium phosphate eluate. This band corresponds to a higher molecular weight protein; however, because of the high concentration of potassium phosphate in the eluted fractions, this salt precipitated after concentrating the fraction using the speed-vac. The high salt concentration slowed the migration of the sample through the gel especially the samples eluted with 100-1000 mM of potassium phosphate.

In order to make sure that this band corresponds to PARP, we had to remove the salt from the hydroxylapatite-eluted fractions. We tried to clean the hydroxylapatite peak using Bio-Spin 30 desalting columns (Bio-Rad). These columns contain Bio-Gel P-30 polyacrylamide gel suspended in 1 ml Tris buffer (10 mM Tris-HCl, pH 7.4) (BioRad Catalog number 732-6231).

In order to remove potassium phosphate from the hydroxylapatite peak, we applied the following procedures:

- 1- The desalting columns were inverted several times to resuspend the settled gel and remove any bubbles.
- 2- The tip was snapped off and the column was placed in a 2.0 ml microcentrifuge tube. The cap was removed and the excess packing buffer allowed to drain by gravity. The drained buffer was discarded and the gel was washed 3 times each with 500  $\mu$ l deionized water. The column was centrifuged for 2 minutes at 1,000 xg to remove the excess water.
- 3- The column was placed in a clean 2.0 ml microcentrifuge tube and the hydroxylapatite peak fraction was applied carefully directly to the center of the column.
- 4- The column was centrifuged for 4 minutes at 1,000 xg.

The sample eluted from the desalting column was concentrated using a speed-vac and resolved together with the NE and the phosphocellulose peak through 10% SDS-PAGE. Unfortunately, we did not detect any band corresponding to PARP in the hydroxylapatite peak (Figure 9) after using the desalting column. There is a possibility that the desalting column retained PARP in addition to the potassium phosphate.

Instead of passing the hydroxylapatite peak through desalting spin columns, the fraction was dialyzed against a buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM KCl, 1.5 mM  $MgCl_2$ , and 1 mM DTT for 3 hours at 4°C. The dialyzed fraction was centrifuged at 10,000 rpm for 5 minutes at 4°C and concentrated using a speed-vac.

The NE, phosphocellulose peak and the concentrated hydroxylapatite peak were resolved through 10% SDS-PAGE. PARP was detected by Western blot analysis using anti-human PARP antibody (1:500, BD Pharmingen). A very clear band



corresponding to PARP was detected in the hydroxylapatite peak (Figure 10). PARP was also highly enriched after this method of purification (Compare the density of PARP band in lane 3 with those in lanes 1 and 2, Figure 10).

### **III. PARP Activity in the Nuclear Fraction (NE) and in the Phosphocellulose Peak:**

PARP assay was performed according to the methods of Jump and Smulson (1980), and Knights and Chambers (2001).

The standard reaction mixture (100  $\mu$ l) contains 50 mM Tris-HCl, pH 8.0, 25 mM MgCl<sub>2</sub>, 1 mM NAD<sup>+</sup>, 10  $\mu$ g activated DNA, and 1-2  $\mu$ l <sup>32</sup>P-NAD (Perkin Elmer Life Sciences, specific activity 1000Ci/mmol) and increasing concentration of NE protein fractions. The reaction mixture was incubated at 25°C for 10 minutes. The poly(ADP-ribosyl)ated proteins were precipitated with 20% ice-cold trichloroacetic acid (TCA). The pellets were then washed with 10% TCA and dissolved in the scintillation fluid followed by liquid scintillation counting. The reactions were carried out in the absence or presence of 3-aminobenzamide (3-AB) which is a known inhibitor of PARP enzymatic activity.

Figure 11 shows the increase in PARP activity with increasing concentration of proteins in the NE fraction. No activity was detected in the presence of 3-AB indicating that the assay conditions are specific for PARP.

Since PARP needs single or double-stranded DNA breaks to be activated, we performed the assay in the absence or presence of activated DNA. As shown in Figure 12, PARP was not active in the absence of activated DNA indicating that the activity of this enzyme is completely dependent on the presence of a damaged DNA.

PARP activity was also measured in the fractions eluted from phosphocellulose column. Figure 13 clearly demonstrates that most of the PARP activity was observed in the phosphocellulose peak (0.5 M NaCl eluted fraction).

We also compared PARP activity in the NE fraction and in the fractions eluted from phosphocellulose column. PARP activity was approximately doubled in the phosphocellulose peak relative to the NE fraction (Figure 14, Compare PARP activity in **10  $\mu$ g** NE protein fraction with that present in only **1  $\mu$ g** phosphocellulose peak).

PARP activity was also determined in the hydroxylapatite peak and the **specific activity** of PARP in that fraction was compared with that in the NE fraction and phosphocellulose peak (Figure 15). The hydroxylapatite peak was used to determine the K<sub>m</sub> and V<sub>max</sub> of PARP isolated from malignant and non-malignant breast cells.

#### **IV. Kinetic Analysis of PARP Purified from Non-Malignant and Malignant Breast Cells:**

This study potentially identified fundamental difference in the activity of PARP in malignant and non-malignant breast cells. PARP was purified from malignant and non-malignant breast cells according to our established procedures using a combination of phosphocellulose and hydroxylapatite chromatography techniques (Section II). The initial rate of incorporation of  $^{32}\text{P}$ -NAD into acid-insoluble material was measured at distinct time interval after initiating the reaction.  $K_m$  and  $V_{max}$  for  $^{32}\text{P}$ -NAD incorporation by PARP were determined from plots of  $1/\text{rate}$  vs  $1/[\text{substrate}]$ .

We started our analysis by performing PARP assay using increasing concentrations of either the purified PARP or  $^{32}\text{P}$ -NAD trying to optimize the conditions for our assay and to make sure that we are using appropriate concentrations of both the enzyme and the substrate.

We performed our kinetic analysis by incubating the reaction mixture (800  $\mu\text{l}$ ) at  $25^\circ\text{C}$ . At different time intervals after initiating the reaction (0, 1, 3, 5, 10, 15, and 30 min), 100  $\mu\text{l}$  were removed from the reaction mixture and transferred to tubes containing 900  $\mu\text{l}$  ice-cold trichloroacetic acid (20%). The tubes were kept in ice until all samples have been processed. The tubes were then centrifuged at 12,000  $\times g$  for 10 minutes at room temperature. The supernatants were removed and discarded. The protein pellets were washed with 1 ml ice-cold trichloroacetic acid (10%) and the supernatant was removed after centrifugation. The protein pellets were dissolved in 1 ml of liquid scintillation cocktail and the  $^{32}\text{P}$ -NAD incorporated into acid-insoluble materials were measured by liquid scintillation.

One problem we have faced during this experiment is that the results were not consistent and  $^{32}\text{P}$  counts were not increasing steadily with increasing the incubation time. One possible explanation for this is the possibility of removing part of the precipitated protein pellets during the washing step with trichloroacetic acid (we might have mistakenly removed part of the pellet during taking off the supernatant after centrifugation step). In order to overcome this problem, we introduced some modification to the assay. After precipitating the poly(ADP-ribosyl)ated protein with 20% ice-cold trichloroacetic acid, we spotted the reaction mixtures on GF/C glass microfiber filters (Whatman) (instead of precipitating protein pellets at the bottom of tubes by centrifugation). The filters were washed with 10% ice-cold acetic acid, dried at room temperature, transferred to scintillation vials, and  $^{32}\text{P}$ -NAD incorporated was measured by liquid scintillation. This modification dramatically improved the results obtained.

Our results indicated that non-malignant breast cell PARP has an apparent  $K_m$  for NAD of 0.15 pmole with a  $V_{max}$  value of  $1.2 \times 10^{-7}$ . PARP isolated from malignant breast cancer cells, however, has a  $K_m$  of 0.23 pmole and the  $V_{max}$  is  $2.7 \times 10^{-7}$  (Figure 16).

## **V. Two Dimensional SDS-PAGE Analysis of PARP from Malignant (MDA MB-468) and Non-Malignant (MCF-10A) Breast Cells:**

In order to determine the isoelectric point (pI) and the 2-D PAGE mobility of PARP isolated from malignant and non-malignant breast cells, the nuclear extracts from MDA MB-468 and MCF-10A cells were prepared and analyzed using Bio-Rad Mini-Protean II tube cells (Bechtel et al., 1998). Approximately, 100 µg of protein were loaded onto the first dimension tube gel containing 9.2 M urea, 4% acrylamide, 20% Triton X-100, 1.6% Bio-Lyte 8/10 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, 0.01% ammonium persulfate, and 0.1% TEMED. The proteins were separated along a pH gradient created using 100 mM NaOH (upper running buffer) and 10 mM H<sub>3</sub>PO<sub>4</sub> (lower running buffer). After the first dimension focusing step, the tube gels were equilibrated in a buffer containing 0.0625 M Tris-HCl, pH 6.8, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, and bromophenol blue. After equilibration, the tube gels were placed onto a 10% SDS-polyacrylamide gel and the proteins were resolved by molecular weight. The proteins were transferred to a nitrocellulose membrane and PARP was detected using anti-human PARP monoclonal antibody.

Since PARP is a basic protein with a pI value > 9.0, we could not separate PARP by this method. In order to solve this problem, we tried two alternatives:

- 1- Using nonequilibrium pH gradient electrophoresis (NEPHGE) according to the method of Anderson (1988) and Prasad et al. (1999). Proteins were loaded on the top of the tube gels and overlaid with 4 M urea to protect proteins from phosphoric acid, which was used in the upper reservoir. The lower reservoir was filled with NaOH. The electrical leads were reversed at the power supply and the gels were run at 400 V for 1 hour followed by 4 hours at 800 V. The tube gels were transferred to 10%SDS-PAGE for resolution in the second dimension.

- 2- Using the Bio-Rad IEF cell.

This cell performs the first dimension isoelectric focusing and can provide a maximum voltage of 10,000 V, which permits better focusing and resolution of the proteins. In addition, the precast immobilized pH gradient gel strips (Bio-Rad ReadyStrip IPG strips) were used in place of the first dimension tube gels. These strips provide reproducible gradients and eliminate the gradient drift that might occur with the tube gels.

PARP in the nuclear fractions have been analyzed using the IEF cell and IPG strips (pH 3-10).

- Approximately 100 µg of nuclear protein fraction were desalted using the Bio-Spin 6 Tris Columns (Bio-Rad). The sample was concentrated using a SpeedVac.
- Prior to isoelectric focusing, the IPG strips were rehydrated with a rehydration buffer containing 8 M urea, 1% CHAPS, 15 mM DTT, 0.1% Bio-Lyte 3/10 ampholyte, and 0.001% bromophenol blue.
- After SpeedVac, the protein sample was dissolved in 135 µl rehydration buffer and loaded in the sample loading well in the focusing tray. The

IPG strip was placed into the channel tray by sliding it through the rehydration solution. After approximately 30 minutes, mineral oil was applied to each channel containing the IPG strip to prevent sample evaporation during the focusing step.

- Prior to running the second dimension, it was necessary to equilibrate the IPG strips. Two equilibration steps (15 min each) were necessary; the first step was required to saturate the strips with SDS and the reducing agent, while the second equilibration step was required to prevent protein re-oxidation during electrophoresis and alkylates the residual DTT to minimize vertical streaking.

Unfortunately, we were not able to detect PARP by using any of the two alternatives. PARP isolated from MDA MB-468 were detected once and the methods were not reproducible.

We tried to re-use the Bio-Rad Mini-Protean II tube cells with some modifications. Instead of using 8/10 and 3/10 Bio-Lyte ampholytes (1.6 and 0.4%, respectively), we prepared the tube gels using **2% of 3/10 Bio-Lyte ampholyte**. By using this Bio-Lyte as the sole ampholyte in the tube gels, we were able to detect PARP in the NE fraction from both MDA MB-468 and MCF-10A cells. This method was reproducible and we obtained the same results by repeating the experiments more than 6 times.

Figure 17 clearly demonstrates that PARP from the breast cancer cells MDA MB-468 exhibits a different migration pattern, when resolved by 2D SDS-PAGE, as compared to the enzyme from non-malignant breast cells; indicating that this protein may be modified in breast cancer cells (Compare PARP migration in the upper and lower panels, Figure 17). The enzyme present in non-malignant breast cells had a basic pI and was resolved as a single spot; however, malignant breast cell PARP appeared as a basic spot in addition to less abundant species having less basic pI values.

In order to check if this modification is due to poly(ADP-ribosyl)ation of the protein, the membrane was stripped off and re-probed with anti-PAR polyclonal antibody (1:1000, Trevigen) that recognizes the poly(ADP-ribose) polymer. We were not able to detect any poly(ADP-ribosyl)ated proteins at the molecular weight region corresponding to that of PARP (116 kDa) (Figure 18). Possible phosphorylation of PARP isoforms detected in breast cancer cells will be tested (see section VII).

## **VI. Improved Two Dimensional SDS-PAGE Resolution of PARP and its Modified Isoforms Using Isoelectric Focusing Cell and IPG Strips:**

Although we were able to detect PARP by using Bio-Rad Mini-Protean II tube cell and Western blot analysis, we could not detect spots corresponding to PARP after staining the second dimension gels with Coomassie blue or silver stain. This might be due to the lower sensitivity of Coomassie or silver staining compared to Western blot. In addition, the tube cell has lower resolution and focusing capacity compared to isoelectric focusing (IEF) cell. The later cell performs the first dimension focusing step at a higher voltage which allows better focusing and resolution of proteins. Therefore, it was necessary to devote much time trying to adjust the conditions required for detection of PARP using IEF cell. Utilization of IEF cell and IPG strip gels to resolve PARP had two major advantages in our project:

- 1- It allowed us to identify spots corresponding to PARP after staining the gel with Coomassie blue, a critical step toward mass spectrometric analysis of PARP.
- 2- The use of IEF cell and IPG strips allowed the resolution of the altered PARP isoform over a wider pH range; an extremely useful breakthrough that subsequently allowed us to detect the nature of post-translational modification of these isoforms (see section VII).

We performed 2D SDS-PAGE using the Bio-Rad IEF cells and IPG ready strips that we have tried in the previous section. Here we introduced two modifications to the procedures that we have used in the previous section.

- 1- We used IPG 7 cm strips (Bio-Rad) with a **pH gradient ranging from 7 to 10** (instead of the IPG strips with pH range 3-10 that we tried previously). This pH range is used to focus and resolve basic proteins with high pI values (theoretical pI of PARP is 8.99).
- 2- After desalting our samples with Pierce desalting spin column (and subsequent speed-vac) and prior to the isoelectric focusing, the proteins were dissolved in a rehydration buffer with a composition exactly similar to the one that we have used before (see the previous section) except that **the Bio-Lyte 3/10 ampholyte was replaced with a Bio-Lyte 7/10 ampholyte** (Bio-Rad).

These two modifications in the procedures were extremely useful in resolving PARP and its modified isoforms in malignant breast cells (Figure 19). The modified PARP isoforms appeared as less abundant spots extending over a wider pH range.

## **VII. Identifying the Post-translational Modification of PARP isoforms in MDA MB-468 Breast Cancer Cells:**

### **1- The modified PARP isoforms are not phosphorylated:**

Because anti-PAR polyclonal antibody did not detect any poly(ADP-ribosyl)ated proteins corresponding to the molecular weight of PARP (116 kDa) (Figure 18), we initiated experiment to test possible phosphorylation of the modified PARP isoforms identified in MDA MB-468 breast cancer cells.

#### **\* Digestion with alkaline phosphatase:**

Calf intestine alkaline phosphatase has been widely used to dephosphorylate both proteins and nucleic acids. This enzyme effectively dephosphorylates proteins containing phosphoserine and phosphothreonine residues.

100 µg of proteins in the NE fraction from MDA MB-468 cells were incubated in the absence or presence of 5, 10, and 20 Units of calf intestine alkaline phosphatase (Fisher BioReagents) at 37°C for 1 hour. Proteins samples were desalted by passing through protein desalting spin columns (Pierce) and concentrated by speed-vac. Proteins were analyzed by 2D SDS-PAGE using the Bio-Rad Mini-Protean II tube cells. No change in PARP migration pattern following treatment of NE fractions with alkaline phosphatase suggesting that the modified PARP isoforms are not serine or threonine phosphorylated (Figure 20).

#### **\* Digestion with lambda protein phosphatase (λ-PPase):**

λ-PPase is a  $Mn^{2+}$ -dependent protein phosphatase with activity towards phosphorylated serine, threonine, and tyrosine residues. We examined the possibility of tyrosine phosphorylation of malignant breast cells PARP isoform by incubating 100 µg of MDA MB-468 cells NE fraction with 400 and 1000 Units of λ-PPase (New England BioLabs) at 30°C for 2 hours. Protein samples were processed as described above and analyzed by 2D SDS-PAGE using the Bio-Rad Mini-Protean II tube cells and Western blot analysis. Figure 21 clearly demonstrated that PARP isoforms are not tyrosine phosphorylated since no change in PARP migration pattern was observed following treatment with λ-PPase.

### **2- Inhibition of PARP enzymatic activity in MDA MB-468 breast cancer cells with 3-aminobenzamide to test possible poly(ADP-ribosylation) of PARP isoforms:**

We have previously shown that anti-PAR polyclonal antibody did not recognize any poly(ADP-ribosyl)ated proteins corresponding to PARP (Figure 18). This might be due to the fact that this antibody does not recognize poly(ADP-ribose) polymers shorter than 50 units long. Therefore, we initiated experiments to examine possible poly(ADP-ribosylation) of the altered PARP isoforms identified in breast cancer cells.

Monolayer cultures of MDA MB-468 cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and split every 3-4 days. When the growing cells reached 70-80% confluency, they were divided into three groups: .

- \* Control untreated group,
- \* 3-Aminobenzamide (3-AB)-treated group (5 mM final concentration),
- \* Ethanol-treated group (ethanol was used as a vehicle for 3-AB). Final ethanol concentration was 0.5%.

The cells were allowed to grow for another 24 hours. They were then washed twice with ice-cold PBS, harvested, and pelleted by low-speed centrifugation. The cell pellets were stored at -80°C till fractionated.

The cells were fractionated to the clear nuclear fraction according to our published procedures (Coll et al., 1996). Briefly, the frozen cell pellets were quickly thawed at 37°C and suspended in a homogenization buffer (200 mM sucrose, 50 mM Tris-HCl, pH 7.4, 5 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM DTT, and 0.1 mM PMSF) with gentle rocking for 30 min at 4°C. The cells were homogenized with a sterile Dounce homogenizer immersed in ice and the nuclear pellets were separated from the cytosolic fraction by low-speed centrifugation. The nuclear pellets were suspended in ice-cold nuclear extraction buffer (400 mM KCl, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl<sub>2</sub>, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, and 0.1 mM PMSF) and were rocked for 2 hours at 4°C. The nuclear extract (NE fraction) was separated by centrifugation at 100,000 g for 1 hour at 4°C and dialyzed against a buffer containing 20 mM Tris-HCl, pH 7.4, 5 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 10% glycerol. The dialyzed NE fractions were divided into 50 µl-aliquots and stored at -80°C.

PARP in NE fractions from the three groups of cells was analyzed by 2D SDS-PAGE using both the Bio-Rad mini protean II tube cell and the IEF cells and IPG 7 cm strips (pH 7-10) as described previously. Changes in PARP isoform migration were not clearly detected using tube gels (Figure 22, upper 3 panels). Surprisingly, upon resolving PARP in NE fractions using the IEF cell, the altered PARP isoforms, detected in both control untreated and ethanol-treated MDA MB-468 cells, were dramatically lost following treatment with 3-AB (Figure 22, lower 3 panels). **This indicates that PARP is most probably poly(ADP-ribosyl)ated in non-estrogen-dependent breast cancer MDA MB-468 cells.**

### **VIII. Nuclear Proteins in Malignant and Non-Malignant Breast Cells are Differentially Poly(ADP-Ribosyl)ated:**

PARP has been found to be poly(ADP-ribosyl)ated in breast cancer cells MDA MB-468 compared to non-malignant MCF-10A cells. We, therefore, examined poly(ADP-ribosyl)ation of nuclear proteins from both cell lines. 50 µg of proteins in NE fraction isolated from both malignant and non-malignant breast cells were resolved through 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and poly(ADP-ribosyl)ated proteins were detected by Western blot analysis using anti-PAR polyclonal antibody (Trevigen). Figure 23 clearly demonstrates that proteins in NE fractions from MDA MB-468 and MCF-10A are differentially poly(ADP-ribosyl)ated.

We have also tried to use the IEF cell and IPG strips (pH 7-10) to detect poly(ADP-ribosyl)ated PARP isoforms using anti-PAR polyclonal antibody (Trevigen). 250 and 500 µg nuclear protein fractions were resolved by 2D SDS-PAGE according to our modified procedures (Section VI). After the second dimension SDS-PAGE, the proteins were transferred to nitrocellulose membranes and poly(ADP-ribosyl)ated proteins were detected by Western blot analysis. Faint spots corresponding to poly(ADP-ribosyl)ated proteins with molecular weights corresponding to that of PARP were detected upon resolving 500 µg of NE protein fraction with IPG strips (pI 7-10) (Figure 24, panel B).

### **IX. Mass Spectrometric Analyses of PARP isolated from Non-Malignant and Malignant Breast Cells**

Samples of 500 µg of proteins from the NE fractions of either MDA MB-468 or MCF-10 cells were resolved by 2D SDS-PAGE according to the procedures adjusted in section V. After the second dimension SDS-PAGE, the gels were fixed in 50% (v/v) methanol and 5% (v/v) acetic acid for 1 hour with gentle shaking. The gels were subsequently stained with GelCode Blue Stain Reagent (Pierce). Imaging was then accomplished using a GS-710 Calibrated Imaging Densitometer (Bio-Rad) and analysis performed by Phoretix 2D Evolution software.

**In-Gel Trypsin Digestion was performed according to the following procedures (Coligan et al., 2000):**

- 1- Spots were excised from the stained polyacrylamide gel and placed in microcentrifuge tubes.
- 2- 100 µl of 25 mM  $\text{NH}_4\text{HCO}_3$ /50% acetonitrile were added and the tubes were mixed for 35-40 min on a low setting using a microtube mixer. The pale blue solution of Coomassie staining was removed and discarded. This step of washing/dehydration was repeated up to 3 times.



- 3- The gel pieces were dehydrated with 100  $\mu$ l acetonitrile. The gel pieces shrunk and became opaque white in color. The acetonitrile was removed and replaced with fresh.
- 4- Finally, the acetonitrile solution was removed and the gel pieces were dried in a speed-vac for 3-5 min.
- 5- The gel particles were rehydrated in 25  $\mu$ l trypsin solution and placed in ice for 10-15 min.
- 6- Excess trypsin solution was removed and the rehydrated gel particles were overlaid with 30  $\mu$ l of 25 mM  $\text{NH}_4\text{HCO}_3$  to keep them immersed through digestion.
- 7- The gel particles were incubated for 12-16 hours at 37°C.
- 8- The tubes were centrifuged for 1 min and the supernatant was collected in a PCR tube.
- 9- The gel pieces were extracted once with 30  $\mu$ l of 25 mM  $\text{NH}_4\text{HCO}_3$ /50% acetonitrile with continuous shaking for 10-15 min at room temperature.
- 10- The tubes were centrifuged briefly and the supernatant transferred to the PCR tube from step 8.
- 11- Extract the gel pieces twice (10-15 min each) with 5% formic acid/50% acetonitrile. After centrifugation, the supernatants are transferred to the PCR tube from step 8.
- 12- The volume of tryptic digest was reduced to 5  $\mu$ l in a speed-vac.
- 13- 15  $\mu$ l of 1% formic acid were added for LC-MS/MS analysis.

#### **Mass Spectrometry:**

The tryptic digests were loaded into a Surveyor AS 3000 autosampler (ThermoFinnigan) then analyzed by reversed-phase high performance liquid chromatography (HPLC) with a Surveyor MS Pump followed by electrospray ionization tandem mass spectrometry (LC-MS/MS) using an LCQAdvantage (ThermoFinnigan) ion trap mass spectrometer fitted with an orthogonal electrospray source equipped with a low flow metal needle. The buffers used for HPLC were 5% acetonitrile/0.2% formic acid in water (buffer A) and 95% acetonitrile/0.2% formic acid in water (buffer B).

Briefly, the samples were loaded into a 20  $\mu$ l loop present on the autosampler and then trapped onto a 0.5  $\mu$ l OPTI-PAK C18 trap cartridge (Optimize Technologies) for desalting and concentration in 100% buffer A. The trapped peptides were then back-flushed onto a 0.1 mm x 150 mm EVEREST C18 capillary column (Vydac) by a linear gradient of 0% to 60% buffer B in 60 min followed by a gradient from 60% to 80% before re-equilibration at flow rates 0.5  $\mu$ l/min. Eluting peptides were analyzed by a data dependent triple play experiment and the resulting collision induced fragmentation (CID) spectra was then searched against known human PARP genes present in both Swissport and NCBI's non-redundant databases using MASCOT and SEQUEST MS/MS search algorithm.

Figure 25 is a Coomassie stain of nuclear proteins from malignant and non-malignant breast cells resolved by 2D SDS-PAGE using IEF cell and IPG strips (7cm, pI

7-10). 14 spots were picked from the two gels and subjected to digestion by trypsin followed by MS/MS analysis. PARP was the main protein identified in spot # 6 (Figure 25B). However, we could not detect any spot corresponding to the modified PARP isoforms. This might be due to the very low abundance of these isoforms. Proteins identified in these spots are shown in table 1:

	Spot #*	Predominant Protein
<b>MCF-10A</b>	1	Heat Shock Protein 90 $\alpha$ (hsp-90 $\alpha$ )
	2	Splicing Factor, praline and glutamine-rich
	3	Splicing Factor, praline and glutamine-rich
	4	Splicing Factor, praline and glutamine-rich
	5	Vinculin
<b>MDA MB-468</b>	6	<b>Poly(ADP-ribose) Polymerase (PARP)</b>
	7	Smcx protein (PARP was present in low abundance)
	8	Heterogenous nuclear riboprotein U (hnRNP U)
	9	Vinculin (PARP was present in low abundance)
	10	Heterogenous nuclear riboprotein M (hnRNP M)
	11	Vinculin
	12	Heterogenous nuclear riboprotein U (hnRNP U)
	13	Vinculin
	14	Vinculin

Table 1: Predominant proteins identified by Mass Spectrometric analysis after in-gel digestion of proteins in spots excised from polyacrylamide gels.

\* Spots 1-5 were from MCF-10A gel and spots 6-14 from MDA MB-468 gel.

Although we could not locate spots corresponding to the altered PARP isoforms in malignant breast cells MDA MB-468 or a spot corresponding to PARP in non-malignant breast cell, the use of IEF cell and IPG strips (pI 7-10) allowed us to better focus and resolve basic proteins. We are planning to use 11 or 17 cm IPG strips (pI 7-10) which will allow us to load up to 3 mg of proteins for Coomassie staining.

#### **X- The Modified PARP Isoforms are present in MCF-7 Breast Cancer as well as Ovarian Cancer Cells:**

We initiated experiments in to test whether the modified isoforms of PARP that were detected in non-estrogen dependent breast cancer cells MDA MB-468 are also present in the estrogen-dependent breast cancer MCF-7 cells. Monolayer cultures of MCF-7 cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine. The cells were harvested and washed 3 times with PBS (8.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, and 1.5 mM KH<sub>2</sub>PO<sub>4</sub>). The cells were then pelleted by low-speed centrifugation (200 g, 5 min, 4°C) and the cell pellets were stored at -80°C until fractionated.

The cell pellets were fractionated to the clarified nuclear extract (NE) according to our published protocol (Coll et al., 1996) (Figure 1). 15 and 30  $\mu$ g of nuclear protein fractions were resolved through 10% SDS-PAGE and Western blot analysis was performed using anti-human PARP monoclonal antibody (BD Pharmingen). Figure 26A (lanes 2 and 3) demonstrates that PARP is present in the NE fraction isolated from MCF-7 cells. In addition to the estrogen-positive breast cancer MCF-7 cells, three ovarian cancer cell lines (PA-1, Hey, and CaOV3) were tested for the presence of the modified forms of PARP. PARP was identified in the NE fraction from the ovarian cancer cells (PA-1 and Hey) (Figure 25A, lanes 4-7). However, we could not detect PARP in the NE fraction isolated from the ovarian cancer cells CaOV3 (Figure 26A, lanes 8 and 9). 2D SDS-PAGE analyses showed that the modified PARP isoforms are present in both MCF-7 and PA-1 cells NE fractions. However, PARP in Hey cells was resolved as a single spot with a basic pI value (Figure 26B).

## **KEY RESEARCH ACCOMPLISHMENTS:**

- Extensive purification of PARP from malignant and non-malignant breast cells (MDA MB-468 and MCF-10A, respectively) using a combination of phosphocellulose and hydroxylapatite chromatography. This step is considered the key step for determining and comparing the kinetic and physical properties of the enzyme isolated from the two cell lines.
- Defining the kinetic properties ( $K_m$  and  $V_{max}$ ) of PARP purified from malignant and non-malignant breast cells.
- Detection of a modified form of PARP in breast cancer cells.
- Utilizing both the Bio-Rad Mini-Protean tube cell and the IEF cell to resolve and identify PARP and its altered isoforms in breast cells. The use of IEF cell allowed better focusing and resolution of PARP isoforms over a wide pH range. This was subsequently used to identify changes in PARP isoforms in response to PARP inhibition with 3-AB.
- Identifying the post-translational modification of PARP isoforms in breast cancer cells.
- Detection of differentially poly(ADP-ribosyl)ated proteins in malignant and non-malignant breast cells.
- Analyzing PARP by tandem MS/MS.
- Detection of the modified PARP isoforms in estrogen-dependent breast MCF-7 cells and the ovarian cancer PA-1 cells.

## **REPORTABLE OUTCOMES:**

### **Abstracts:**

- 1- Abdel-Aziz W, Han S, Hickey RJ, and Malkas LH (2002). Purification and functional characterization of breast tumor cell poly(ADP-ribose) polymerase. Presented at the *Era of Hope* department of Defense Breast Cancer Research Meeting, Orlando, FL, September 25-28.
- 2- Abdel-Aziz W, Hoelz D, Malkas LH, and Hickey RJ (2002). Purification and functional characterization of breast cancer cell poly(ADP-ribose) polymerase. Presented at the 25<sup>th</sup> Annual San Antonio Breast Cancer Symposium, San Antonio, TX, December 11-14.
- 3- Abdel-Aziz W, Malkas LH, Hoelz D, and Hickey RJ (2003). Proteomic analysis of breast cancer cell poly(ADP-ribose) polymerase. Presented at the 94<sup>th</sup> AACR Annual Meeting, Washington DC, July 11-14.
- 4- Abdel-Aziz W, Malkas L, Hoelz D, and Hickey R (2003). The molecular "nick sensor" protein poly(ADP-ribose) polymerase is modified in breast cancer cells. Presented at the AACR-NCI-EORTC International Conference on *Molecular Targets and Cancer Therapeutics*, Boston, MA, November 17-21.
- 5- Abdel-Aziz W, Malkas LH, Hoelz D, and Hickey RJ (2004). A modified form of poly(ADP-ribose) polymerase is present in breast cancer cells. Presented at the 95<sup>th</sup> AACR Annual Meeting, Orlando, FL, March 27-31.
- 6- Adel-Aziz W, Malkas LH, Hoelz D, Loehrer AP, and Hickey RJ (2004). Defining the structure and function of an altered form of poly(ADP-ribose) polymerase identified in breast cancer cells. Accepted for presentation at the 27<sup>th</sup> Annual San Antonio Breast Cancer Symposium. San Antonio, TX, December 8-11.

### **Research Articles:**

- Abdel-Aziz W, Malkas LH, Hoelz D, and Hickey RJ (2004). Proteomic and functional analysis of breast cell poly(ADP-ribose) polymerase. Submitted

## **CONCLUSIONS:**

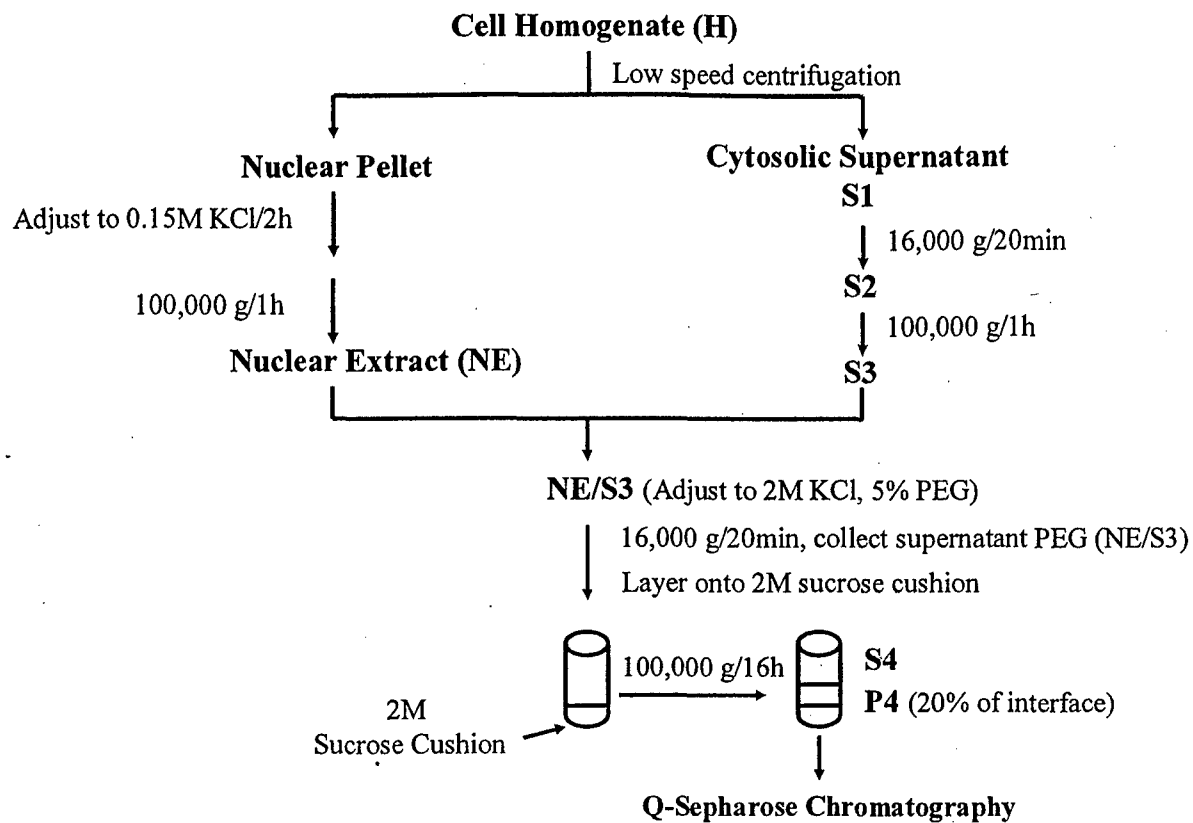
- PARP is confirmed by SDS-PAGE and mass spectrometric analyses to be one of the components of the DNA synthesome.
- The combination of the hydroxylapatite with phosphocellulose chromatography techniques resulted in extensive purification and enrichment of PARP.
- Two-dimensional SDS-PAGE analysis of PARP indicated the presence of altered isoforms of this protein in breast cancer cells (MDA MB-468 and MCF-7).
- The modified PARP isoforms identified in breast cancer cells are not phosphorylated. These altered isoforms are probably due to poly(ADP-ribosyl)ation (PARP automodification).
- In addition to breast cancer cell lines (MDA MB-468 and MCF-7), the modified PARP isoforms are also detected in ovarian cancer PA-1 cell line.
- Nuclear proteins from malignant and non-malignant breast cells are differentially poly(ADP-ribosyl)ated suggesting different role of PARP in breast cancer.
- Mass spectrometry was successfully used to identify PARP after 2D SDS-PAGE analysis of nuclear proteins and subsequent Coomassie staining followed by in-gel digestion with trypsin.
- PARP isolated from malignant breast cells has different kinetic properties ( $K_m$  and  $V_{max}$ ) compared to those of non-malignant PARP.

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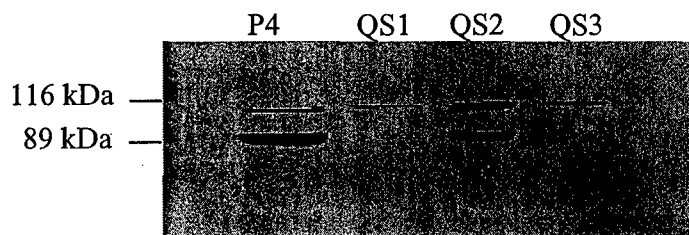
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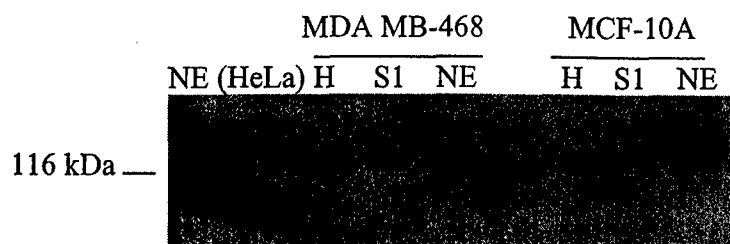




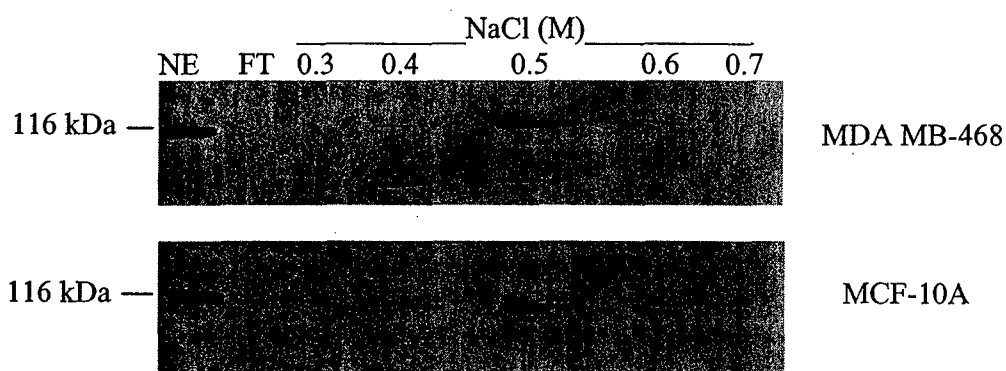
**Figure 1:** Flow Diagram of Subcellular Fractionation Scheme



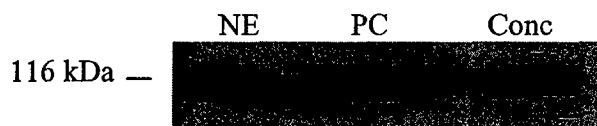
**Figure 2:** Immunoblot analysis of PARP in P4 and Q-Sepharose fractions



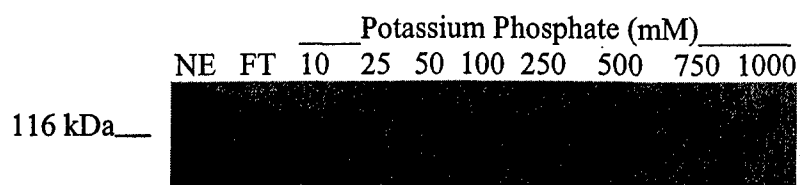
**Figure 3:** Immunoblot analysis of PARP in the homogenate (H), cytosolic (S1) and nuclear (NE) fractions from MDA MB-468 and MCF-10A cells. 50  $\mu$ g proteins from each fraction were resolved through 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-PARP monoclonal antibody (1:500, BD Pharmingen). NE protein fraction from HeLa cells were resolved in a parallel lane and was used as a positive control.



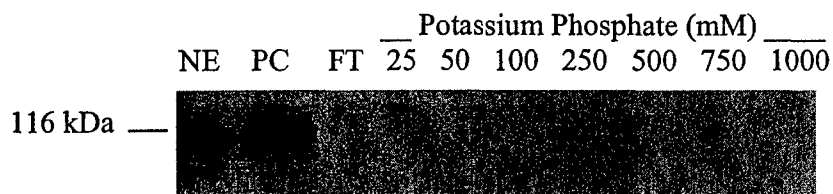
**Figure 4:** Immunoblot analysis of PARP in the NE, flow throw (FT), and in the fractions eluted from phosphocellulose column with increasing concentrations of NaCl (0.3-0.7 M).



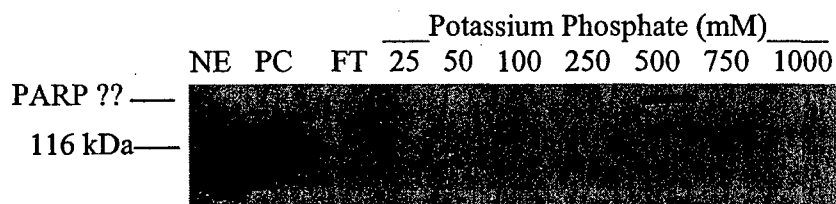
**Figure 5:** Immunoblot analysis of PARP in the NE fraction, Phosphocellulose peak (fraction eluted with 0.5 M NaCl, PC) and after concentration of the PC peak using Microcon centrifugal devices.



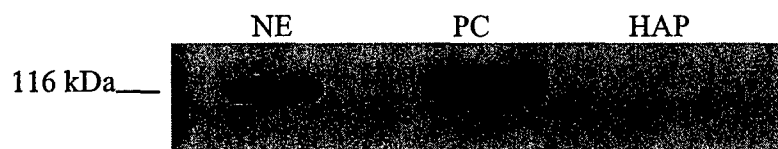
**Figure 6:** Immunoblot analysis of PARP in NE, FT, and in the fractions eluted from hydroxylapatite column with increasing concentrations of potassium phosphate buffer (10 mM-1 M).



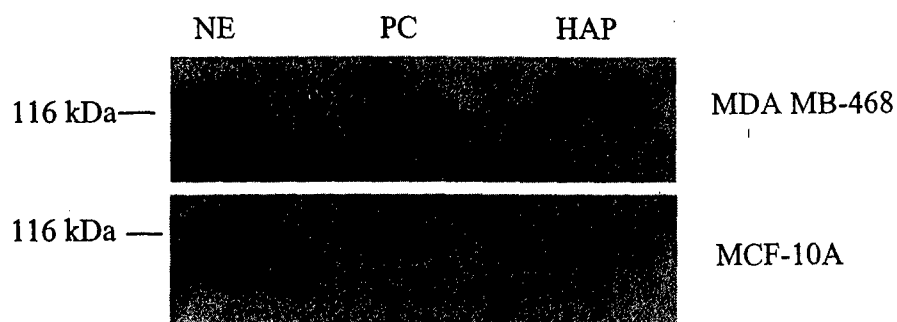
**Figure 7:** Immunoblot analysis of PARP in NE, PC peak, FT, and in fractions eluted from hydroxylapatite column with a stepwise increasing concentration of potassium phosphate buffer (25 mM-1 M).



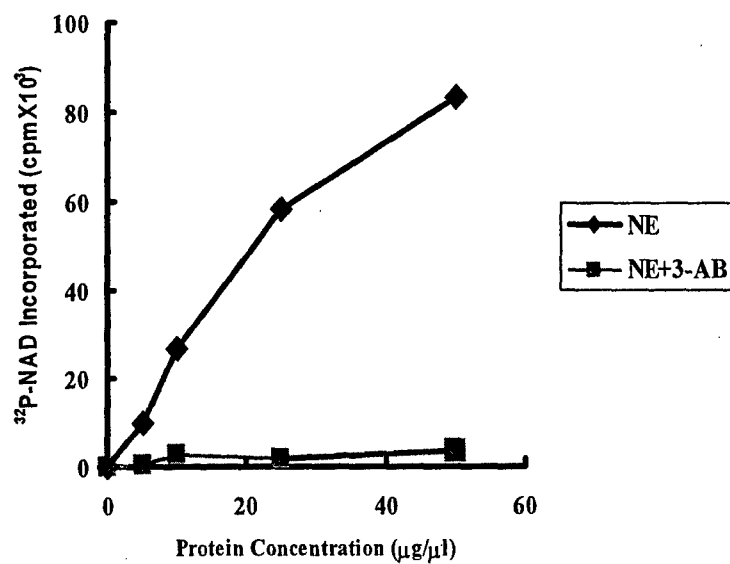
**Figure 8:** Immunoblot analysis of PARP in NE, PC, FT, and in the fractions eluted from hydroxylapatite column after concentration using a speed-vac.



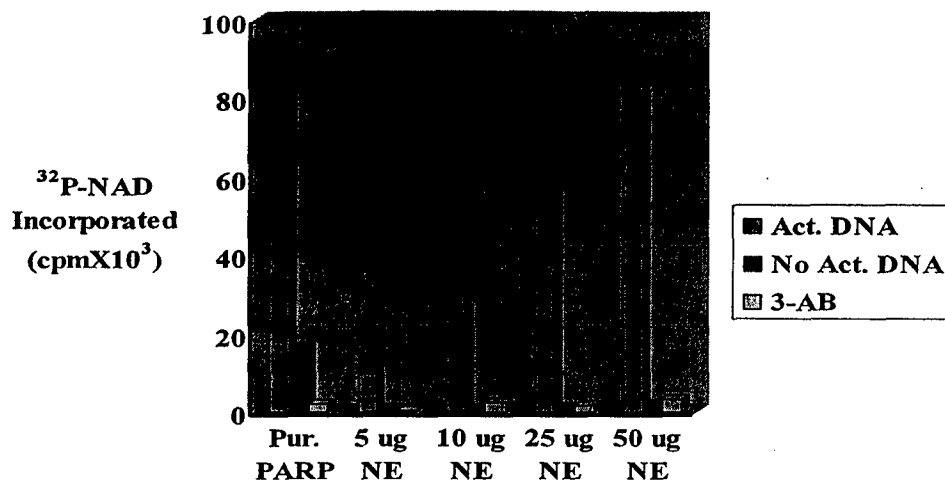
**Figure 9:** Immunoblot analysis of PARP in NE, PC, and hydroxylapatite peak (HAP, fraction eluted with 500 mM potassium phosphate) after applying the HAP onto the BioRad desalting column and speed-vac.



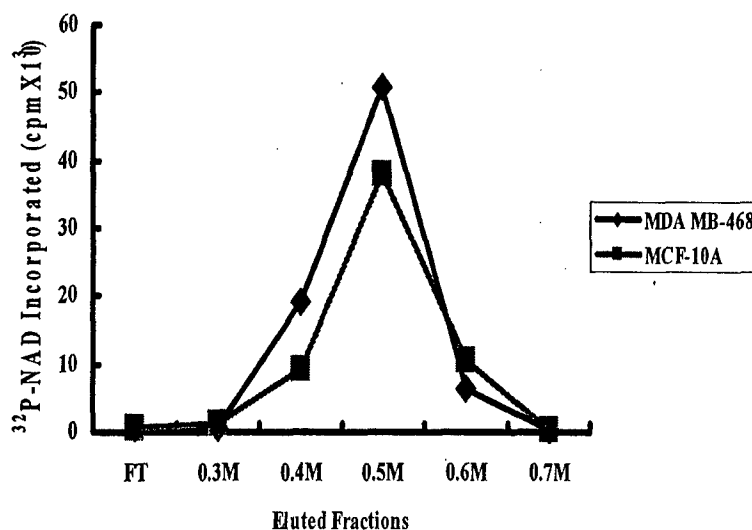
**Figure 10:** Immunoblot analysis of PARP in NE, PC, and HAP after dialysis of the later fraction for 3 hours at 4 °C, with 2 changes of the dialysis buffer followed by speed-vac.



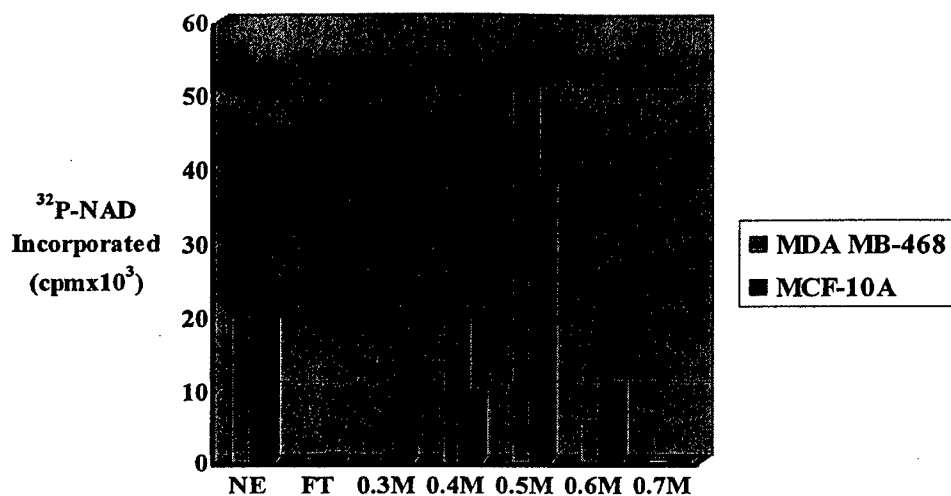
**Figure 11:** PARP activity in NE fraction in the absence or presence of 3-AB.



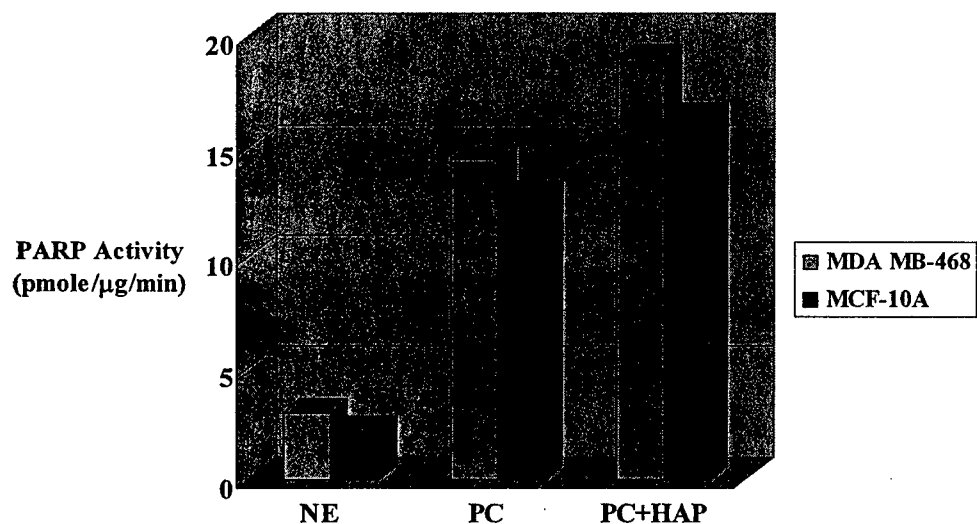
**Figure 12:** PARP activity in NE fraction isolated from breast cancer cells (MDA MB-468) in the absence or presence of activated DNA and in the absence or presence of 3-AB. The activity of the enzyme is completely dependent on the presence of DNA strand breaks and is inhibited in the presence of 3-AB. The same experiment was carried out using 20 µg of partially purified enzyme (Trevigen) for comparison.



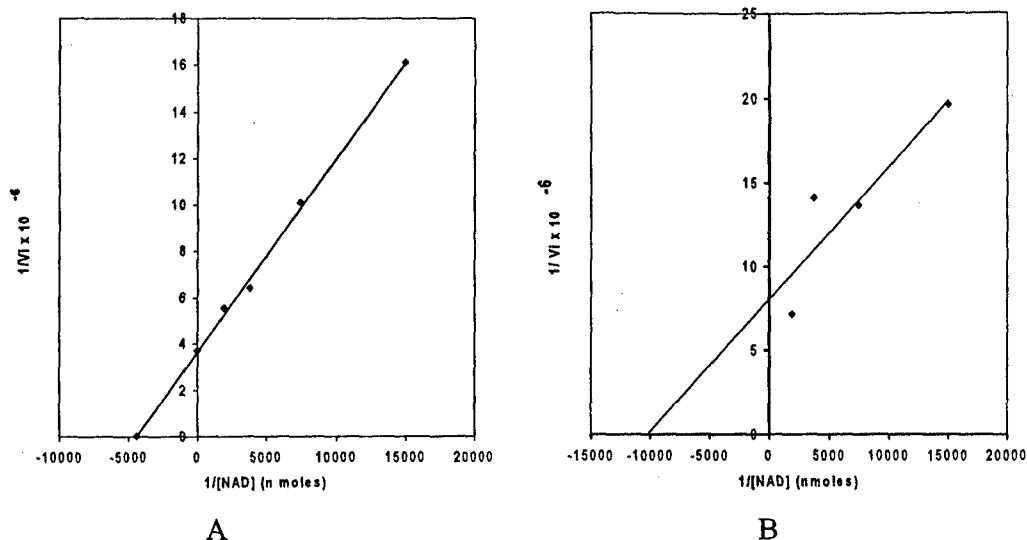
**Figure 13:** PARP activity in the FT and in fractions eluted from phosphocellulose with increasing concentrations of NaCl.



**Figure 14:** Enrichment of PARP enzymatic activity after its partial purification using phosphocellulose chromatography.



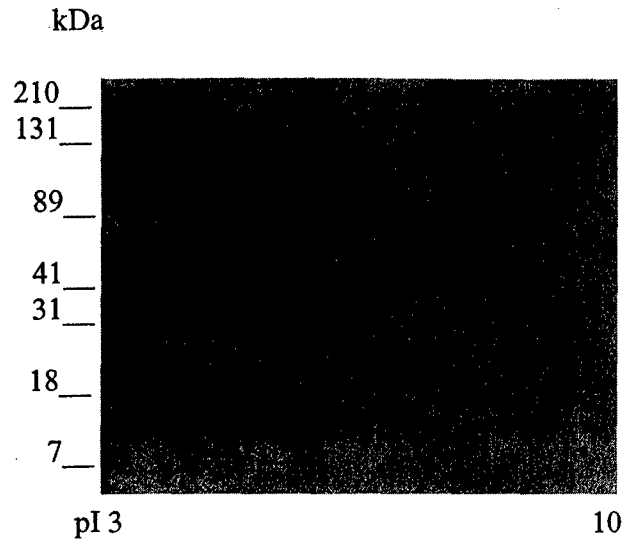
**Figure 15:** PARP activity in NE, PC peak, and after its extensive purification with a combination of phosphocellulose and hydroxylapatite (PC+HAP) chromatography.



**Figure 16:** Kinetic analysis of PARP in malignant (A) and non-malignant breast cells.



**Figure 17:** 2D SDS-PAGE analysis of PARP in MDA MB-468 and MCF-10A cells. 100  $\mu$ g of NE protein fraction were loaded onto the first dimension tube gels and proteins were separated along a pH gradient created using 100 mM NaOH (upper running buffer) and H<sub>3</sub>PO<sub>4</sub> (lower running buffer). The tube gels were placed onto 10% SDS-polyacrylamide gels and PARP was detected by Western blot analysis using anti-PARP monoclonal antibody (1:500, BD Pharmingen).

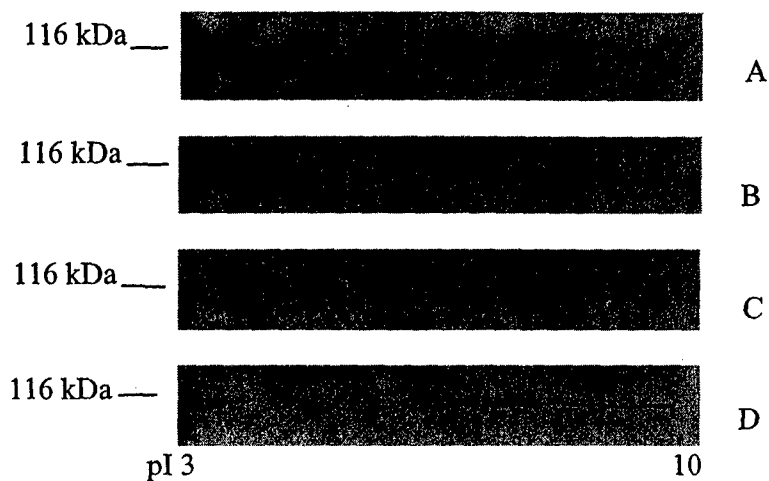


**Figure 18:** Testing possible poly(ADP-ribosyl)ation of PARP isoforms identified in breast cancer cells. The nitrocellulose membrane from the previous experiment (Figure 11) was stripped off and reprobed with anti-PAR polyclonal antibody (1:1000, Trevigen) that recognizes the poly(ADP-ribose) polymer.

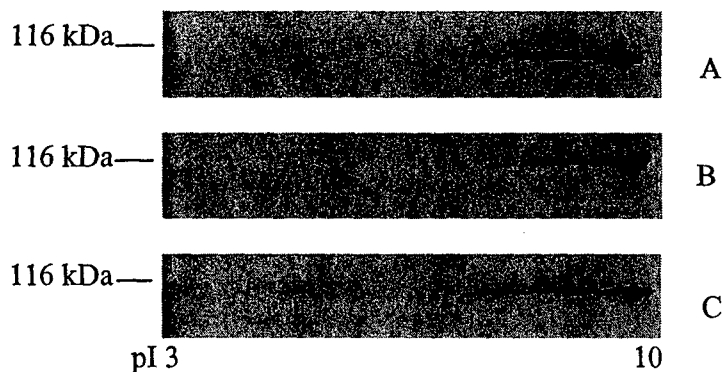


**Figure 19:** 2D SDS-PAGE analysis of PARP in NE fraction of non-malignant (MCF-10A) and malignant (MDA MB-468) cells using IPG 7cm strips (pH 7-10) and isoelectric focusing cell.

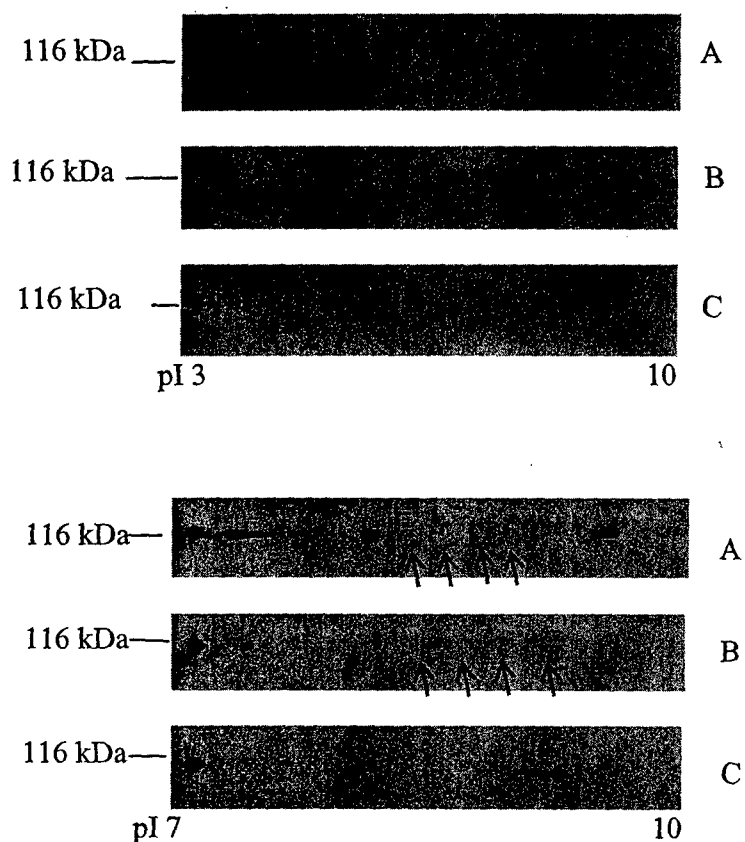




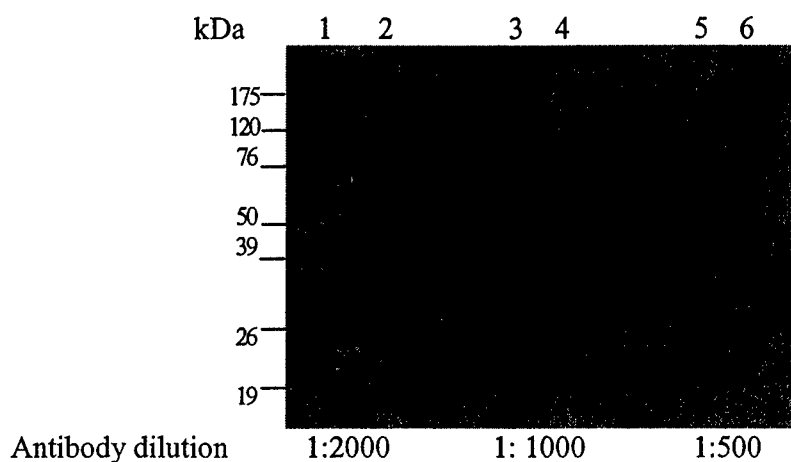
**Figure 20:** 2D SDS-PAGE analysis of PARP in control (A) untreated NE fractions and after treatment with 5 (B), 10 (C), or 20 (D) units of calf intestine alkaline phosphatase.



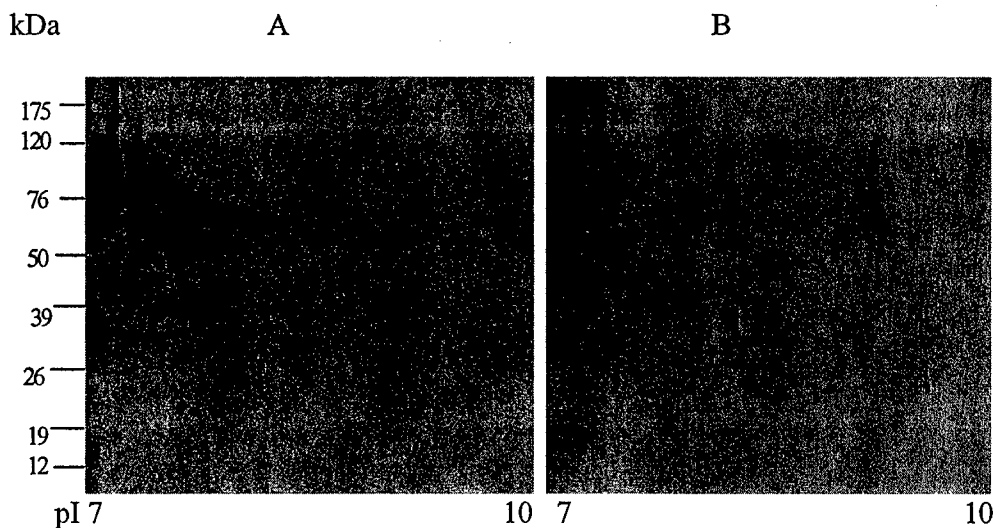
**Figure 21:** 2D SDS-PAGE analysis of PARP in control NE fraction (A) and following treatment with 400 (B) or 1000 Units of  $\lambda$ -PPase.



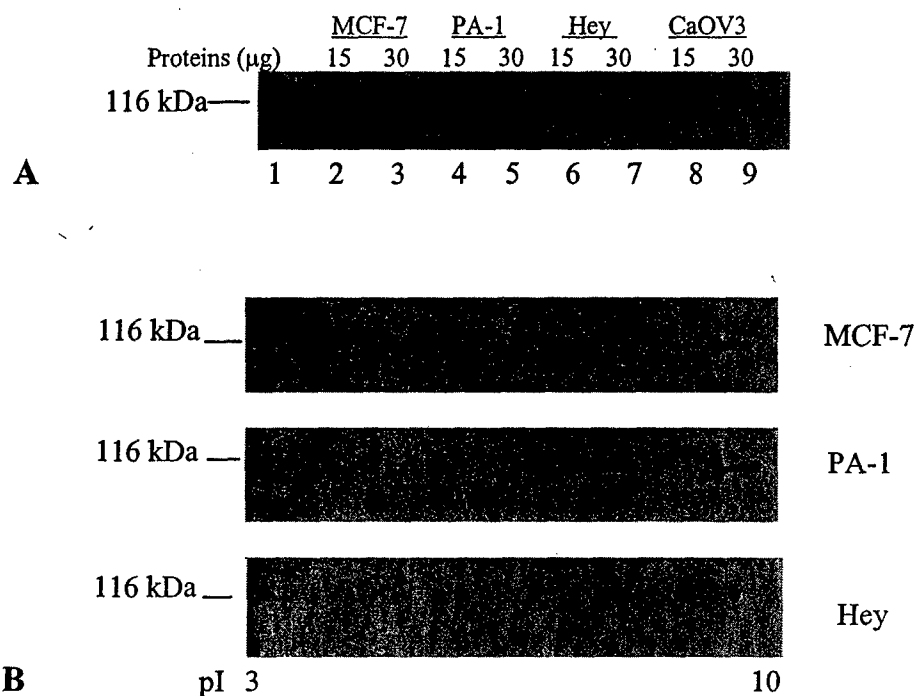
**Figure 22:** 2D SDS-PAGE analysis of PARP in NE fraction from control untreated (A), ethanol-treated (B), and 3-AB-treated MDA MB-468 cells. The arrows indicate the position of altered PARP isoforms that were lost after inhibiting PARP enzymatic activity with 5 mM 3-AB. PARP and its isoforms were resolved by tube gels (upper 3 panels) and IEF cells (lower 3 panels).



**Figure 23:** Poly(ADP-ribosyl)ation of nuclear proteins in malignant MDA MB-468 (lanes 1, 3, 5) and non-malignant MCF-10A (lanes 2, 4, 6) cells. Western blot analysis was performed using different dilutions of anti-PAR rabbit polyclonal antibody (Trevigen).



**Figure 24:** 2D SDS-PAGE analysis of poly(ADP-ribosyl)ated proteins in MDA MB-468 cells. 250 (A) or 500 (B) µg of NE proteins were analyzed using IEF cells and IPG strips (7cm, pI 7-10). After the second dimension SDS-PAGE, proteins were transferred to nitrocellulose membranes and poly(ADP-ribosyl)ated proteins detected by Western blot analysis using anti-PAR polyclonal antibody (1:500, Trevigen).



**Figure 26:** **A.** Immunoblot analysis of PARP in MCF-7, PA-1, Hey, and CaOV3 cells. 15 and 30  $\mu$ g of NE protein fractions from each cell were resolved through 10% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes and PARP was identified using anti-PARP monoclonal antibody (BD Pharmingen). 20  $\mu$ g of MDA MB-468 NE fraction were resolved in a parallel lane and was used as a positive control. **B.** 2D SDS-PAGE analyses of PARP in MCF-7, PA-1, and Hey NE fraction using the Bio-Rad Mini-Protean II tube cells.